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(54) Title: GROWTH FACTOR RECEPTOR (57) Abstract <p>A 75-85 Kd glycopolypeptide, capable of acting as a receptor for polypeptide of the bombesin type, is isolated from the surface of Swiss 3T3 cells. Certain antagonists and antibodies to the glycopolypeptide are described, the antagonists being of quite different structure to bombesin e.g. [D-Pro²]-spantide, [D-Phe⁵]-spantide and position 5 variants thereof. The antagonists and antibodies are of interest medically in that they are able to influence cell proliferation that occurs under the influence of the bombesin-like polypeptides.</p>		

GROWTH FACTOR RECEPTOR

THIS INVENTION relates to antagonists and antibodies to growth factor receptors and is particularly concerned with antagonists and antibodies to bombesin receptors.

5 The amphibian tetradecapeptide bombesin (6) and mammalian peptides structurally related to bombesin, which include gastric releasing peptide (GRP) and the neuromedins (7 to 12) are growth factors which are believed to be implicated in the control of cell proliferation. Bombesin-like peptides are present in high concentrations in small cell lung carcinoma (18 to 21) where they could act as autocrine growth factors (22). The bombesin group of peptides interact in the cell with receptors but while a certain amount is known about the chemistry of the bombesin group of peptides, very little is known about the chemistry of the receptors of the bombesin-like peptides. In view of the involvement of the bombesin-like peptides in cell growth and the implications on cell growth of the presence or absence of bombesin/receptor interactions, a detailed study of the receptors is clearly of importance.

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We have now developed methods that have enabled us to identify certain receptors to certain peptides of the bombesin family which enables them to be characterised as new compounds and it is with such receptors and,

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additionally, to antagonists and antibodies to such receptors, that the present invention is directed. The bombesin family of peptides do have structural differences from one another but also have a common 7-amino acid sequence and we believe that the receptor we have identified is capable of acting as receptor to various members of the bombesin family, regardless of the species of origin of the bombesin-like peptide.

In one aspect, the present invention provides a polypeptide having the following characteristics:

1. It is a single chain glycopolypeptide, having at least two mannose side chains.
2. It binds selectively with polypeptides of the bombesin type.
3. It has a molecular weight of 75 to 85 Kilodaltons (Kd).
4. It has an isoelectric point of 6.4 to 6.9.
5. Its core protein, obtained using endo-beta-N-glucosaminidase from Flavobacterium meningosepticum, has a molecular weight of about 42Kd.
6. It binds with Antagonist A and Antagonist D, both as hereinafter defined.

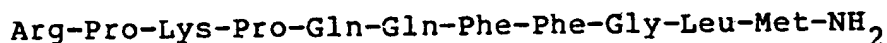
In a further aspect, we provide antagonists to the glycopolypeptide receptors as defined above. These antagonists are substances which are structurally quite different to bombesin and the bombesin-like peptides but

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which can bind to the bombesin receptor, and inhibit the effects of bombesin, but we believe, not by occupying binding sites that would otherwise be occupied by the bombesin-like peptides. Since bombesin-like peptides have been identified as present in high concentration in small cell lung carcinoma (18-21), and may act as autocrine growth factors (22), bombesin antagonists are of interest in providing a means of interfering with the receptor/bombesin peptide interaction and hence cell growth patterns influenced by the bombesin-like growth factor. We have now shown that an antagonist of bombesin that we have identified can inhibit the growth of human small cell lung cancer (SCLC) lines and is therefore of interest as a promising therapeutic entity.

Antibodies against the receptor are of similar interest in that they too can occupy sites on the receptor that might otherwise be occupied by bombesin-like peptides and similarly, may be able to influence the cell growth pattern.

So-called Substance P is an 11-mer neuropeptide, of interest in studies in pain transmission, which has the formula:



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We have found that a commercially available structural variant of Substance P, known as [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹] Substance P and hence of the formula:

5 D-Arg-D-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH₂

which we call Antagonist A and which is also known as [D-Pro²]spantide, is, surprisingly, able to act as a bombesin antagonist. Antagonist A is therefore of interest, according to the present invention, in the
10 modification of cell growth influenced by the presence of bombesin-like peptides.

We have also found that a further commercially available structural variant of Substance P, which we call Antagonist D, which is also known as [D-Phe⁵]spantide and
15 which has the formula:

D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH₂

is even more potent as a bombesin antagonist than Antagonist A and hence is also of great interest for the modification of cell growth influenced by the presence of
20 bombesin-like peptides.

Our studies of Antagonists A and D indicate to us the importance of structural variation at amino acid

position 5 in compounds of this type to produce improved bombesin antagonists and the present invention extends to new compounds which are amino acid position 5 variants on Antagonist A and Antagonist D and to such position

- 5 5-variants of Antagonist A and Antagonist D for use in method of treatment of the human or animal body by therapy or in a method of diagnosis carried out on the human or animal body.

Suitable position 5 variants are:-

D-Trp, D-Tyr and Me-Phe

A further feature of the present invention comprises antibodies against the bombesin receptors. The antibodies can be raised against the receptor by conventional methods which include the step of injecting a
15 vertebrate with the polypeptide receptor of the invention as immunogen and can be polyclonal antibodies or monoclonal antibodies.

Polyclonal antibodies against the receptor can be raised by conventional methods involving immunisation
20 of an animal with the receptor molecule in immunogenic form followed by recovery of polyclonal antibodies from blood fragments of the immunised animal.

Monoclonal antibodies against the receptor can be generated by conventional procedures involving the

immunisation of test animals such as mice with the receptor in immunogenic form followed by hybridisation of spleen cells from the immunised mice with myeloma cells to give hybridoma cell lines secreting monoclonal antibodies
5 against the receptor.

The polyclonal or monoclonal antibodies generated in these ways can be purified by affinity chromatography against a solid phase carrying receptor bearing cells, for example, by a procedure which include
10 the steps of bringing the antibody into contact with a solid phase bearing a polypeptide receptor of the invention or bearing Swiss 3T3 cells carrying on their surface a polypeptide receptor of the invention to form an antibody/antigen conjugate and releasing purified antibody
15 from the conjugate.

The antagonists or antibodies of the invention may be formulated with pharmaceutically acceptable carriers or diluents e.g. conventional parenteral carriers so that the antagonists or antibodies can be administered
20 parenterally where they are of interest for use in a method of treatment of the human or animal body for diagnosis or therapy, and more specifically in the diagnosis or therapy of cancers where uncontrolled cell growth is associated with disorders of proteins of the
25 bombesin family. The antagonists and antibodies are also of interest for use in the production of a medicament for the treatment of uncontrolled cell proliferation.

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The antagonists or antibodies of the invention are also of interest for use in the in vitro diagnosis of uncontrolled cell proliferation by a method which includes the step of bringing the antagonist or antibody into
5 contact with a body sample from a host suspected of suffering from uncontrolled cell proliferation. The method is particularly useful in the diagnosis of cancers by histology or serum assay.

The isolation and molecular characterisation of
10 the receptors of the invention requires a procedure for their identification and we have developed a system involving the use of certain bifunctional cross-linking reagents which have already been used to identify membrane receptors in other systems (23 to 28). We have used the
15 cross-linking agent ethylene glycol bis(succinimidyl succinate) to link covalently ^{125}I labelled gastrin releasing peptide (^{125}I -GRP) to a surface protein in Swiss 3T3 cells. This surface protein, when isolated from the Swiss 3T3 cells, displays the characteristics of a
20 specific receptor for the peptides of the bombesin family. This protein was not present in other cell lines which do not exhibit receptor properties for the bombesin-like peptides.

More specifically, the polypeptide receptor of
25 the invention can be isolated by a process which comprises incubating a culture of Swiss 3T3 cells in a culture

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medium including ^{125}I labelled gastrin releasing peptide (^{125}I -GRP), further incubating the ^{125}I -GRP treated Swiss 3T3 cells in the presence of a bifunctional crosslinking reagent and solubilising the resulting ^{125}I -GRP/
5 crosslinking reagent/polypeptide conjugate to release the polypeptide from the cell surface.

Swiss 3T3 cells are widely available for experimental use and are available from the American Type Culture Collection in Rockville, Maryland, U.S.A., under
10 the Deposit No. ATCC-CCL92.

The following Examples are given to illustrate the isolation and characterisation of the receptor of the present invention.

EXAMPLE 1Materials and MethodsMaterials

Bombesin and litorin were obtained from Sigma. GRP, the 14-27
5 amino acid fragment of GRP and [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]
substance P were obtained from Bachem Fine Chemicals (Saffron Walden,
U.K.) and the 1-16 fragment of GRP and neuromedin B were from
Peninsula Laboratories (San Carlos, CA). Highly purified
platelet-derived growth factor (PDGF) was obtained from Bioprocessing.
10 Ethylene glycol bis (succinimidylsuccinate) (EGS), disuccinimidyl
suberate (DSS), dithio-bis (succinimidylpropionate) (DSP) and bis
[2-(succinimidocarbonyloxy)ethyl] sulphone (BSCOES) were purchased
from Pierce Chemical Co. ¹²⁵I-GRP (2000 Ci/mmol; 1Ci = 37 GBq) was
obtained from the Radiochemical Centre (Amersham, U.K.) or was
15 prepared by radiolabelling GRP with ¹²⁵I using the soluble
lactoperoxidase method (29,30). The labelled peptide was separated
from unreacted Na ¹²⁵I as described (8). ¹²⁵I-GRP exhibited mitogenic
activity within a similar concentration range to that observed with
the unlabelled peptide. All other reagents used were of the highest
20 grade available.

Chemical cross-linking of ¹²⁵I-GRP to receptors

Confluent and quiescent cultures of Swiss 3T3 cells were incubated
at 15°C in 1ml of medium consisting of 0.14 M NaCl, 5 mM KCl, 0.01 M
Na₂HPO₄, 1.8 mM KH₂PO₄, 1.8 mM CaCl₂, 1 mM MgCl₂, 25 mM
25 4-(2-hydroxy thyl)-1-piperazin ethane sulphonic acid (binding medium)

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pH 7.0, supplemented with 0.1% BSA and the appropriate concentration of ^{125}I -GRP in the presence or absence of a 500-fold excess of unlabelled GRP. After 2.5 h the cells were washed three times at 15°C with phosphate buffered saline (PBS) and then incubated for 15 min at 15°C in 1 ml of binding medium, pH 7.4 in the presence of the appropriate cross-linking agent at the concentration indicated. The cross-linking agents (EGS, DSS, DSP and BSCOE) were dissolved in dimethyl sulphoxide immediately prior to use and were added to medium to give a final concentration of dimethylsulphoxide of 1-2%. The cultures were rapidly rinsed twice with PBS at 4°C and solubilized in 0.1 ml of 2 x sample buffer 0.2 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 6% sodium dodecyl sulphate (SDS) (w/v) 4% β -mercaptoethanol (v/v) and 2 mM ethylenediaminetetraacetic acid. Samples were immediately heated at 100°C for 3-5 min and analysed by either one or two-dimensional gel electrophoresis.

SDS-polyacrylamide gel electrophoresis

Slab gel electrophoresis was performed using 7.5% acrylamide in the separating gel and 5% in the stacking gel, and 0.1% SDS (31). After electrophoresis gels were stained, destained and dried down onto paper for autoradiography with Fuji X-ray film (Fuji Photo Film Co. Ltd. Japan). Dried gels were exposed to film for 4-8 days. Two-dimensional gel electrophoresis was performed as described by O'Farrell (32) using isoelectric focusing in the 1st dimension and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8% polyacrylamide) in the second dimension. The samples prepared for isoelectric focusing containing 1.4% LKB ampholytes, pH 5-7, plus 0.6% LKD

ampholytes, pH 3.5-10, 6M urea, and 2% Nonidet P-40. The Mr 75000-85000 band from autoradiograms was scanned using a Joyce-Loebl double beam densitometer and the areas under specific peaks were measured with a Hewlett-Packard digitizer.

5 Cell culture procedure (33), assays of DNA synthesis by ³H-thymidine incorporation (34) and ¹²⁵I-GRP binding to intact cells (13) were performed as described previously.

Results and Discussion

When cultures of confluent and quiescent Swiss 3T3 cells were incubated with ¹²⁵I-GRP at 15°C cell-associated radioactivity reached a maximum after 2.5 h (Fig. 1A) and was considerably enhanced compared with surface binding at either 37°C or 4°C (unpublished observations). Analysis by SDS-PAGE of cells which were incubated with 5nM ¹²⁵I-GRP at 15°C for 2.5 h, and treated with the homobifunctional cross-linking agents EGS, DSS, DSP or BSCOEES revealed the presence of a single major band of Mr 75000-85000 (Fig. 1B). Identical results were obtained using either commercially available ¹²⁵I-GRP or GRP radiolabelled with ¹²⁵I in our laboratory. In the presence of 500-fold excess unlabelled GRP(+) this band was completely abolished. EGS displayed the greatest efficiency of cross-linking and this agent was therefore used in subsequent experiments. The effect of EGS on the level of the Mr 75000-85000 protein was concentration-dependent; the half-maximal effect of EGS was obtained at a concentration of 2 mM (Fig. 1C). The rank order of cross-linking efficiency (EGS>DSS>DSP>BSCOEES) may be related to the chain length of the arms of these bifunctional molecules. Thus BSCOEES which has the shortest chain length was

virtually ineffectiv (Fig. 1B). The Mr 75000-85000 protein appeared as a multicomponent spot migrating with an isoelectric point of 6.4 to 6.9 when cross-linked cultures of Swiss 3T3 cells were analysed by two-dimensional gel electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension (results not shown).

The Mr 75000-85000 band was not obtained when the cross-linking reaction was carried out either in the absence of the cross-linking agent, in plastic dishes without cells, or using other cell lines including Rat-1, whole mouse embryo fibroblasts and Balbc/3T3, which neither exhibit significant specific ^{125}I -GRP binding, nor respond mitogenically to bombesin-related peptides (13). The possibility that the affinity labelled band was a degradation product of a higher molecular weight protein was tested by extracting cultures after the cross-linking reaction in the presence of the protease inhibitors aprotinin (100 $\mu\text{g}/\text{ml}$), pepstatin (4 $\mu\text{g}/\text{ml}$), phenylmethylsulphonylfluoride (2 mM) and ethyleneglycol-bis-(β -aminoethyl ether)N,N'-tetra-acetic acid (4 mM). These treatments had no significant effect on the level of the Mr 75000-85000 protein and did not result in the appearance of any higher molecular weight proteins. In another experiment performed at 4°C to prevent ligand internalization and degradation identical results were obtained to those shown in fig. 1 (results not shown). Thus, the Mr 75000-85000 protein was neither an intracellular component associated with internalized ^{125}I -GRP or a product of peptide degradation, nor a fragment arising from proteolysis of a larger molecule. In addition, treatment with 0.6 M 2-mercaptoethanol did not result in the appearance of additional bands of lower molecular weight suggesting that the Mr 75000-85000 protein consists of a single polypeptide

chain. These important controls strongly suggested that the Mr 75000-85000 band was a surface component of Swiss 3T3 cells closely related to the receptor for peptides of the bombesin family.

The above conclusion was further substantiated by cross-linking ^{125}I -GRP to cultures incubated in the presence of different concentrations of unlabelled peptide. The decrease in the level of the Mr 75000-85000 band with increasing concentrations of unlabelled GRP (Fig. 2A, open symbols) closely paralleled the ability of GRP to inhibit the binding of an identical concentration of ^{125}I -GRP in a parallel set of cultures (Fig. 2A, closed symbols). The cross-linking of ^{125}I -GRP to Swiss 3T3 cells was also markedly inhibited by other peptides structurally related to GRP including bombesin, neuromedin B, litorin and the bombesin antagonist [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹] substance P (13,17,35,36). The amino-terminal fragment of GRP (GRP(1-16)) which neither inhibits ^{125}I -GRP binding, nor stimulates DNA synthesis (13) caused no reduction in the level of the Mr 75000-85000 protein (Table 1). To ascertain the specificity with which ^{125}I -GRP recognizes the Mr 75000-85000 protein, Swiss 3T3 cells were incubated with ^{125}I -GRP in the presence of a variety of other mitogens for these cells. As shown in Table 1, the level of the protein obtained by treatment with EGF was not substantially affected by saturating concentrations of PDGF, epidermal growth factor (EGF), vasopressin, insulin, and phorbol 12,13-dibutyrate (1,37,38). In addition the neuropeptides substance P, substance K and somatostatin also had no effect on affinity labelling of the Mr 75000-85000 band (Table 1). This result is in accord with the finding that the binding of ^{125}I -GRP to intact 3T3 cells is also not inhibited by these mitogens (13).

The conclusion that the Mr 75000-85000 protein is a major component of the receptor for peptides of the bombesin family in Swiss 3T3 cells was further strengthened by measuring the level of the protein as a function of the concentration of the radioiodinated ligand. Fig. 2B shows that the cross-linking of ^{125}I -GRP to the Mr 75000-85000 band increased in a saturable manner with increasing concentration of the labelled peptide. A double-reciprocal plot of these data (not shown) produced a straight line and gave a value for the K_d of 1 nM which compares very favourably with the K_d ($0.5 \times 10^{-9}\text{M}$) obtained from Scatchard analysis of binding curves (13). Furthermore, the dependence of affinity labelling of the Mr 75000-85000 protein on ^{125}I -GRP concentration closely parallels the ability of the peptide to stimulate a variety of early biological responses (16,17) and DNA synthesis (13) in quiescent Swiss 3T3 cells. At high concentrations of ^{125}I -GRP a higher molecular weight band of approximately Mr 160,000 was observed (Fig. 2B). This band represented 4% of the total cross-linked material and was almost undetectable either at lower levels of ^{125}I -GRP or at 4°C.

Footnote¹ The abbreviations used are: GRP, gastrin releasing peptide; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; EGS, ethyleneglycolbis (succinimidylsuccinate); DSS, disuccinimidyl suberate; DSP, dithio-bis-(succinimidylpropionate); BSCOES, bis[2-(succinidooxycarbonyloxy)ethyl]sulphon; SDS-PAGE, sodium dodecyl sulphate-polyacrylamid gel electrophoresis.

EXAMPLE 2

This illustrates the more than fivefold potency of Antagonist D over Antagonist A.

[DPhe⁵]spantide is a potent inhibitor of GRP-mediated mitogenesis

Substance P has a slight amino acid sequence homology with bombesin (Table 2) and neither inhibits the binding of GRP to Swiss 3T3 cells nor stimulates DNA synthesis. However, [DPro²]spantide (Antagonist A, Table 2) which was synthesized as a substance P antagonist was found to be a bombesin antagonist in pancreatic acinar cells and to block the growth-promoting effects of bombesin in Swiss 3T3 cells. In order to identify a more potent antagonist of bombesin-like peptides, we have tested ten substance P antagonists at 50 μ M (Table 2) for their ability to inhibit mitogenesis stimulated by GRP (the mammalian homologue of bombesin in Swiss 3T3 cells). [DPhe⁵]spantide (Antagonist D) was clearly the most potent GRP antagonist. In contrast, peptides, B, C, E, F, G, H, J and K were less potent than either A or D. Spantide (B) had no antagonist activity even at 100 μ M. None of the peptides stimulated DNA synthesis when tested at 20 μ M with insulin at 1 μ g/ml i.e. none exhibited any agonist activity.

Following the identification of [DPhe⁵]spantide as the most promising GRP antagonist, we compared the potency of [DPhe⁵]spantide with that of [DPro²]spantide. Figure 3 shows that [DPhe⁵]spantide at 20 μ M markedly increased the concentration of GRP required to produce half-maximal stimulation of DNA synthesis whereas addition of [DPro²]spantide also at 20 μ M had only a slight effect. Inhibition of DNA synthesis by [DPhe⁵]spantide was completely reversed by high concentrations of GRP, indicating that its inhibitory effect was competitive and reversible. The dose response curves for the two antagonists in the presence of GRP 3.6 nM are shown in Figure 3 (right). Half-maximal inhibition of DNA synthesis was obtained with 22 μ M [DPhe⁵]spantide and 118 μ M [DPro²]spantide. Thus, [DPhe⁵]spantide is 5.4-fold more potent than [DPro²]spantide in inhibiting DNA synthesis induced by GRP.

The following Examples demonstrate that Antagonist D inhibits all of the events initiated by Bombesin/GRP in a specific manner.

EXAMPLE 3

[DPhe⁵]spantide binds competitively to the GRP receptor

The preceding results demonstrate that [DPhe⁵]spantide is a potent inhibitor of GRP-induced DNA

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synthesis which exhibits specificity against other mitogens. To elucidate its mechanism of action, we examined the effect of [DPhe⁵]spantide on the specific binding of [¹²⁵I]GRP to Swiss 3T3 cells. Figure 4 (left) shows that both [DPro²]spantide and [DPhe⁵]spantide caused a concentration-dependent inhibition in the specific binding of [¹²⁵I]GRP (1 nM). Half-maximal inhibition of binding was achieved with 2.3 μ M of [DPhe⁵]spantide and 14 μ M of [DPro²]spantide, a 6.1-fold difference in potency. This is consistent with the relative potencies of the two antagonists in inhibiting DNA synthesis induced by GRP.

The binding of different concentrations of [¹²⁵I]GRP was measured in the absence and presence of 10 μ M [DPhe⁵]spantide. A double reciprocal plot of these data (Figure 4, center) shows that [DPhe⁵]spantide markedly reduces the affinity of the receptors of [¹²⁵I]GRP, although the number of binding sites is unchanged. This is consistent with results previously obtained with [DPro²]spantide and strongly suggests that these peptides bind competitively to the GRP receptor.

To further substantiate these findings, we investigated the effects of the two antagonists on the affinity-labelling of the recently described Mr 75000-85000 protein which is a putative bombesin receptor

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(Figure 4, right). They were both able to differentially inhibit the Mr 75000-85000 protein obtained by cross-linking [125 I]GRP to Swiss 3T3 cells with EGS. Half-maximal inhibition (obtained by scanning densitometry of the autoradiographs) was achieved with [DPhe⁵]spantide at 5.5 μ M and [DPro²]spantide at 20 μ M, again demonstrating the superiority of [DPhe⁵]spantide.

EXAMPLE 4

[DPhe⁵]spantide inhibits the early events elicited by GRP

One of the earliest events stimulated by addition of bombesin or GRP to quiescent Swiss 3T3 cells is an increase in cytosolic Ca²⁺ concentration ([Ca²⁺]_i). Figure 5 (left) shows that the rise in [Ca²⁺]_i caused by the addition of GRP (1 nM) to quiescent Swiss 3T3 cells was prevented by the addition of [DPro²]spantide at 20 μ M but not at 5 μ M. In contrast [DPhe⁵]spantide was effective at 5 μ M, demonstrating that [DPhe⁵]spantide is at least 4-fold more potent than [DPro²]spantide in this assay. These effects were specific and reversible since [DPhe⁵]spantide at 5 μ M did not prevent a response to PDGF and because the effects of the antagonist in preventing Ca²⁺ mobilization in response to 5 nM GRP was reversed by addition of GRP at 50 nM.

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Inhibition of [125 I]EGF binding by GRP, which is mediated by the protein kinase C pathway, was reversed in a concentration-dependent fashion by [DPro²]spantide and [DPhe⁵]spantide (Figure 5, right). Half-maximal reversal of inhibition was obtained with [DPhe⁵]spantide at 8.7 μ M and [DPro²]spantide at 30 μ M. These findings further substantiate the conclusion that [DPhe⁵]spantide is a potent GRP antagonist.

EXAMPLE 5

In addition to the demonstration of inhibition of the cellular effects of Bombesin/GRP in mouse 3T3 cells we have now shown that Antagonists A and C can inhibit the growth of SCLC cells in a specific and reversible fashion.

SCLC is known to secrete bombesin-like peptides which have been suggested to act as autocrine growth factors. Thus it is plausible but as yet unproven that an antagonist to bombesin/GRP will inhibit SCLC growth, so we have now tested the effects of [DPro²]spantide and [DPhe⁵]spantide on SCLC in vitro.

Figure 6 shows that the rate of growth of the SCLC lines H69, H128 and H417 in serum-free medium was abolished by addition of [DPro²]spantide at 150 μ M, a concentration that reversibly inhibits GRP-induced mitogenesis in Swiss 3T3 cells. The inhibition of growth by the antagonist in SCLC cell lines was reversed by

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washing the cells and resuspending them in serum-free medium.

The effects of [DPro²]spantide and [DPhe⁵]spantide on H69 cells are compared in Figure 7. The cells achieve 10-fold increase in number in about 12 days in serum-free medium (inset). Both antagonists inhibited growth in a dose-dependent manner; half-maximal effect was seen at 24 μ M and [DPhe⁵]spantide and at 82 μ M with [DPro²]spantide. The difference in potency is of the same magnitude as that demonstrated in Swiss 3T3 cells and supports the contention that bombesin-like peptides (or vasopressin) may be important growth factors for SCLC.

Figure Legends

Figure 1: A. Time-dependence of ^{125}I -GRP binding to Swiss 3T3 cells at 15°C. Cultures were washed and then incubated at 15°C in 1 ml binding medium containing ^{125}I -GRP (2 nM). After various times the cells were rapidly washed four times with cold (4°C) PBS supplemented with 1 mg/ml bovine serum albumin (BSA) and then incubated with 1 ml 0.2 M acetic acid, 0.5 M NaCl at 4°C for 6 min to remove cell-surface associated ligand. This medium was then removed for counting and the remaining intracellular cell-associated radioactivity extracted with 1 ml 0.1 M NaOH containing 2% Na_2CO_3 and 1% SDS. Open squares denote surface bound ligand and closed squares intracellular radioactivity.

B. ^{125}I -GRP affinity labelling of an Mr 75000-85000 cellular protein using disuccinimidyl cross-linking agents. Confluent cultures of Swiss 3T3 cells were washed and then incubated with ^{125}I -GRP (5 nM) in the presence (+) or absence (-) of 0.9 μM unlabelled GRP for 2.5 h at 15°C. The cells were then rinsed to remove free ligand and treated with either EGS, DSS, DSP or BSOES at concentrations of 6 mM, 2 mM, 2 mM and 4 mM respectively. The cells were solubilized in SDS sample buffer and electrophoresed on a 7.5% polyacrylamide gel. The arrow indicates the position of the Mr 75000-85000 protein. All other experimental procedures used in this and similar experiments were as described in Materials and Methods. A broad and intensely radioactive band migrating at low molecular weight was observed in all our gels. This material was not obtained in the presence of excess unlabelled GRP and therefore is most likely to represent the unreacted peptide.

C. Concentration-dependence of the effect of EGS on the affinity-labelling of the Mr 75000-85000 protein. Confluent cultures of Swiss 3T3 cells were incubated with 1 nM ^{125}I -GRP at 15°C and then treated with various concentrations of EGS as indicated. Samples were prepared for SDS PAGE as described in Materials and Methods. The arrow indicates the position of the Mr 75000-85000 protein. In this and other experiments the efficiency of cross-linking using EGS ranged from 5 to 10% of cell-surface associated radioactivity.

Figure 2: A. Effect of varying concentrations of unlabelled GRP on the ^{125}I -GRP binding to intact Swiss 3T3 cells (●) and the affinity labelling of the Mr 75000-85000 protein (○). Confluent cultures of 3T3 cells were incubated with 5 nM ^{125}I -GRP in the presence of varying concentrations of non-radioactive ligand at 15°C for 2.5 h. After this time some cells were washed four times with cold (4°C) PBS supplemented with 1 mg/ml BSA, solubilized and total cell associated radioactivity was then determined in a gamma counter. Parallel cultures were rinsed with PBS at 15°C and then treated with EGS (6mM) to cross-link bound ^{125}I -GRP. The cells were then solubilized and samples were analyzed by SDS-PAGE. After electrophoresis, gels were dried, exposed to film and scanned as described in Materials and Methods. The areas under the individual peaks for the Mr 75000-85000 protein are shown as a function of the concentration of the unlabelled peptide and are expressed as a percentage of the control. The inset shows the region of the autoradiogram used for the scanning. B. Concentration-dependence of ^{125}I -GRP affinity labelling of the Mr 75000-85000 protein. Confluent cultures of Swiss 3T3 cells were

incubated with different concentrations of ^{125}I -GRP at 15°C for 2.5h, and treated with EGS (6 mM). The level of the Mr 75000-85000 protein expressed in arbitrary units is shown as a function of the concentration of the labelled ligand. Inset: autoradiogram of the gel used for scanning; the Mr 75000-85000 and 160,000 bands are indicated by arrows. All other experimental details were as described in Materials and Methods.

Figure 3. Inhibition of GRP-induced DNA synthesis by [DPro²]spantide (A) and [DPhe⁵]spantide (D). Left: Confluent and quiescent cultures of Swiss 3T3 cells in 35 mm plastic dishes were washed twice with DMEM then incubated at 37°C in 2 ml of a 1:1 mixture of DMEM/Waymouth medium containing [^3H]thymidine at 1 $\mu\text{Ci/ml}$ (1 μM), insulin at 1 $\mu\text{g/ml}$ and increasing concentrations of GRP in the absence (■) or presence of antagonist A at 20 μM (○) or antagonist D at 20 μM (Δ). After 40 hours DNA synthesis was estimated by [^3H]thymidine incorporation into acid-precipitable material. Values are expressed as a percentage of [^3H]thymidine-incorporation obtained with a saturating level of GRP (36 nM) in the absence of antagonist (100% = 8.4×10^5 cpm per dish). Each point represents the mean of duplicate determinations. Right: Cultures of Swiss 3T3 cells were washed and incubated as above, except that the concentration of GRP was fixed at 3.6 nM with various concentrations of antagonists A (○) and D (Δ). Values are expressed as a percentage of [^3H]thymidine incorporation obtained in the absence of antagonists (100% = 8.6×10^5 cpm per dish). Each point represents the mean of 4 determinations.

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Figure 4. Effects of [DPro²]spantid (A) and [DPhe⁵]spantid (D) on the binding of ¹²⁵I-labelled GRP ([¹²⁵I]GRP) to Swiss 3T3 cells.

Left: Inhibition of specific [¹²⁵I]GRP binding by antagonists A and D. Confluent and quiescent cells were washed twice with DMEM then incubated at 37°C in 1 ml of binding medium (3) containing [¹²⁵I]GRP at 1 nM and various concentrations of antagonist A (o) and D (Δ). Cell-associated [¹²⁵I]GRP binding was measured after 30 minutes. Values are expressed as percentage of the specific binding obtained in the absence of antagonists. Non-specific binding was determined by the addition of 300-fold excess of unlabelled GRP. Each point represents the mean of 3 determinations.

Center: Effect of antagonist D on the affinity of binding sites in Swiss 3T3 cells for [¹²⁵I]GRP. Confluent and quiescent cells were washed twice with DMEM then incubated for 30 minutes at 37°C in 1 ml of binding medium containing various concentrations of [¹²⁵I]GRP in the absence (●) or presence (Δ) of D at 10 μM. Specific binding (B) is expressed in pmol/10⁶ cells and is shown in a double-reciprocal plot. Each point represents the mean of duplicate determinations.

Right: Effects of antagonists A and D on the affinity labelling of the bombesin receptor-associated Mr 75000-85000 protein. Confluent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated at 15°C in 1 ml of binding medium (pH 7.0) (15) containing 0.5 nM [¹²⁵I]GRP and various concentrations of the antagonists. After 2 hours, the cultures were washed twice with binding medium then incubated in 1 ml containing 6 mM ethylene glycol bis (succinimidylsuccinate) (EGS) at pH 7.4 for 15 minutes at 15°C. The cultures were then washed twice with cold phosphate-buffered saline and solubilised in 0.1 ml of 2x sample buffer, then immediately heated at 100°C for 5 minutes and electrophoresed on a 10% polyacrylamide gel.

Figure 5. Effects of [DPro²]spantide (A) and [DPhe⁵]spantide (D) on the early cellular responses stimulated by GRP. Left: Effects of antagonists on $[Ca^{2+}]_i$. Quiescent Swiss 3T3 cells grown on Cytodex 2 beads were washed twice with DMEM and incubated for 10 minutes with fura-2 tetracetoxymethyl ester at 1 μ M, then washed three times and suspended in 2 ml of electrolyte solution (6) in the fluorimeter at 37°C and stirred. Fluorescence was recorded continuously in a Perkins-Elmer LS5 luminescence spectrometer with an excitation wavelength of 335 nm and emission wavelength of 510 nm. After a period of equilibration, the following additions were made: solvent, S; antagonist A at 5 μ M, A, 5 and 20 μ M, A, 20; antagonist D at 5 μ M, D, 5. After 3 minutes in each case, GRP was added at 1 nM, G or at 50 nM, G, 50; PDGF at 1 nM, P. Right: Antagonists A and D reverse the inhibition of [¹²⁵I]EGF binding induced by GRP. Confluent and quiescent cultures of Swiss 3T3 cells were washed twice with DMEM then incubated for 1 hour at 37°C in 1 ml of binding medium (9) containing [¹²⁵I]EGF at 0.2 nM and GRP at 3.6 nM alone (■) or in the presence of various concentrations of A (○) or D (Δ). Values are expressed as percentages of the specific binding obtained with [¹²⁵I]EGF alone at 0.2 nM. The non-specific binding was obtained by the addition of 500-fold excess of unlabelled EGF. Each point represents the mean of 6 determinations.

Figure 6. [DPro²]spantide reversibly inhibits the growth of SCLC cell lines. Stock cultures of cell lines H69, H128 and H417 were maintained in RPMI 1640 medium with 10% fetal bovine serum (heat inactivated) in a humidified atmosphere of 10% CO₂:90% air at 37°C.

They were passaged every 7 days. Identical growth was obtained in the serum-free medium of RPMI 1640 supplemented with HITES (29) (hydrocortisone, 10 nM; insulin, 5 µg/ml; transferrin, 100 µg/ml; 17β-estradiol, 10 nM; sodium selenite, 30 nM) and 0.25% bovine serum albumin. Cells were washed twice with RPMI 1640 medium then incubated in the serum-free medium in the absence (●) or presence (□,■) of [DPro²]spantide at 150 µM. After 4 days they were again washed twice with RPMI 1640 medium then resuspended at a density of 5×10^4 cells per ml in the absence (●,□) or presence (■) of [DPro²]spantide at 150 µM (Day 0). Cell number was determined at intervals over 14 days in a Coulter Counter after disaggregation of cell clumps by syringing through 19G and 21G needles. Each point represents the mean of 3 determinations.

Figure 7. Inhibition of SCLC growth in vitro by [DPro²]spantide and [DPhe⁵]spantide is concentration-dependent. Cultures of H69 cells were washed twice with RPMI 1640 medium then incubated in serum-free medium (as in Figure 6) in the absence (●) or presence of various concentrations of [DPro²]spantide (○,△) and [DPhe⁵]spantide (△,D). Cell number was determined in a Coulter Counter after disaggregation of cell clumps by syringing through 19G and 21 G needles. Samples were incubated for 13 days, when the controls (inset) had achieved 10-fold increase in number, indicated by the arrow. Each point represents the mean (\pm standard deviation) of 5 determinations.

Table 1
Specificity of 125 I-GRP affinity labelling
of the Mr 75000-85000 protein

	Addition	M _r 75000-85000 protein
5		% of control
	-	100
	GRP	0.8
	GRP (14-27)	0.4
	Bombesin	0.5
10	Litorin	2
	Neuromedin B	10
	[D-Arg ¹ , D-Pro ² , D-Trp ^{7,9} , Leu ¹¹]	9.7
	Substance P	
	GRP (1-16)	93
15	PDGF	99
	EGF	94
	Vasopressin	94
	Insulin	90
	Phorbol 12,13-dibutyrate	99
20	Substance P	91
	Substance I	94
	Somatostatin	97
	Neur tensin	93

Confluent ultures f Swiss 3T3 cells w re incubated with 125 I-GRP

(0.5 nM) at 15°C for 2.5 h in the absence (-) or presence of the following GRP-related peptides at the concentrations indicated: GRP (360 nM), the 14-27 amino acid fragment of GRP (GRP (14-27)) (30 nM), bombesin (30 nM), litorin (92 nM), neuromedin B (220 nM), the bombesin antagonist [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹] substance P (100 nM) and the 1-16 amino acid fragment of GRP (GRP (1-16)) (3230 nM).

Parallel cultures were incubated with the same concentration of ¹²⁵I-GRP in the presence of the following unrelated factors: PDGF (5

nM), EGF (83 nM), vasopressin (1000 nM), insulin (1 µg/ml), phorbol

12,13 dibutyrate (2000 nM), substance P (740 nM), substance K (880

nM), somatostatin (610 nM) and neurotensin (600 nM). After the

incubation period cultures were treated with 6 mM EGS as described in

Materials and Methods. Each value is expressed as a percentage of the level of the Mr 75000-85000 protein obtained with no additions (-).

Other experimental details are as described in Materials and Methods.

Arg - Gln - Leu - Gly - Asn - Gln - Trp - Ala - Val - Gly - His - Leu - Met - NH₂

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DArg - DPro - Lys - Pro - Gln - Gln - DArg- Phe - DArg- Leu - Leu - NH₂

DArg - Pro - Lys - Pro - Gln - Gln - DTrp- Phe - DTrp -Leu -Leu -NH₂

Arg - DPro - Lys - Gln - Gln - DPro- Phe - DTrp -Leu -Met -NH₂

D Arg - Pro - Lys - Pro - Dphe - Gln - DTrp- Phe - DTrp -Leu -Leu -NH₂

Arg - DPro - Lys - Pro - Gln - DTrp - Phe - DTrp - Leu - Met - NH₂

F DPro - Gln - Gln - DPro - Phe - DPro-DPro -¹H₂

Arg - Lirp - Mepe - Lirp - Leu - Val - m₂

H
Darg - DPro - Lys - Pfo - Gin - DPrE - PHe - DHis - Leu - Met - Val

HAfg - gIY - gIn - gIp-PIe - gIY -Asp -v/vvdu/2

Pro - Gin - Glp - Phe - Asp - Leu - Met - Thr₂

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CLAIMS

1. A polypeptide having the following characteristics:

1. It is a single chain glycopolypeptide, having at least two mannose side chains.

5 2. It binds selectively with polypeptides of the bombesin type.

3. It has a molecular weight of 75 to 85 Kilodaltons (Kd).

4. It has an isoelectric point of 6.4 to 6.9.

10 5. Its core protein, obtained using endo-beta-N-glucosaminidase from Flavobacterium meningosepticum, has a molecular weight of about 42Kd.

6. It binds with Antagonist A and Antagonist D, both as hereinbefore defined.

15 2. A process for the isolation of a polypeptide as defined in claim 1 which comprises incubating a culture of Swiss 3T3 cells in a culture medium including ^{125}I labelled gastrin releasing peptide (^{125}I -GRP), further incubating the ^{125}I -GRP treated Swiss
20 3T3 cells in the presence of a bifunctional crosslinking reagent and solubilising the resulting ^{125}I -GRP/

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crosslinking reagent/polypeptide conjugate to release the polypeptide from the cell surface.

3. A process according to claim 2 wherein the crosslinking reagent is ethylene glycol bis(succinimidyl succinate).

4. A process according to claim 2 or 3 wherein the conjugate is separated from associated impurity by gel electrophoresis.

5. A position 5 variant of Antagonist A or Antagonist D.

6. A position 5 variant according to claim 5 wherein position 5 is substituted by D-Trp, D-Tyr or Me-Phe.

7. An antibody to a polypeptide as defined in claim 1.

8. A monoclonal antibody according to claim 7.

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9. A method of producing an antibody as defined in claim 7 or 8 which includes the step of injecting a vertebrate with a polypeptide as defined in claim 1 as the immunogen.

5 10. A method of purifying an antibody as defined in claim 7 or 8 which include the steps of bringing the antibody into contact with a solid phase bearing a polypeptide as defined in claim 1 or bearing Swiss 3T3 cells carrying on their surface a polypeptide as
10 defined in claim 1 to form an antibody/antigen conjugate and releasing purified antibody from the conjugate.

11. A pharmaceutical composition comprising a position 5 variant according to claim 5 or 6 or an antibody according to claim 7 or 8 together with a
15 pharmaceutically acceptable carrier or diluent.

12. Antagonist A or Antagonist D or a position 5 variant thereof as defined in claim 5 or 6 or an antibody as defined in claim 7 or 8 for use in a method for the treatment of the human or animal body by therapy
20 or in a method of diagnosis.

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13. Antagonist A or Antagonist D or a position 5 variant thereof as defined in claim 5 or 6 or an antibody as defined in claim 7 or 8 for use in the production of a medicament for the treatment of 5 uncontrolled cell proliferation.

14. A method for the in vitro diagnosis of uncontrolled cell proliferation which includes the step of bringing into contact with one another a body sample from a host suspected of suffering from uncontrolled cell 10 proliferation and Antagonist A or Antagonist D or a position 5 variant thereof as defined in claim 5 or 6 or an antibody according to claim 7 or 8.

15. A method for the treatment of uncontrolled cell growth in a host which comprises administering 15 parenterally to the host in need of treatment an effective amount of Antagonist A or Antagonist D or a position 5 variant thereof as defined in claim 5 or 6 or an antibody as defined in claim 7 or 8.

Fig.1.

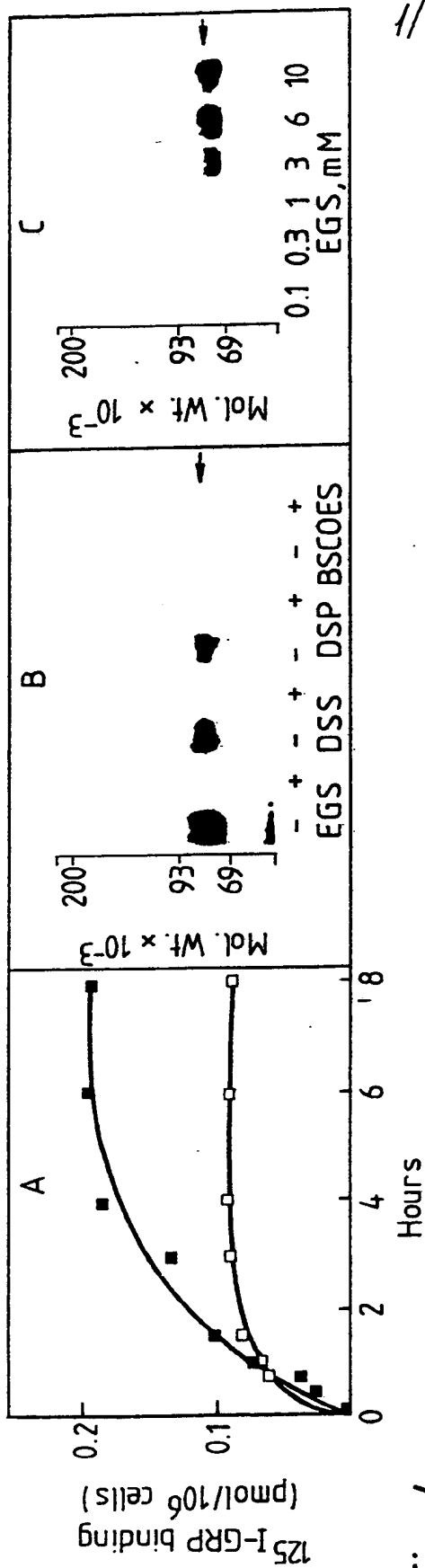
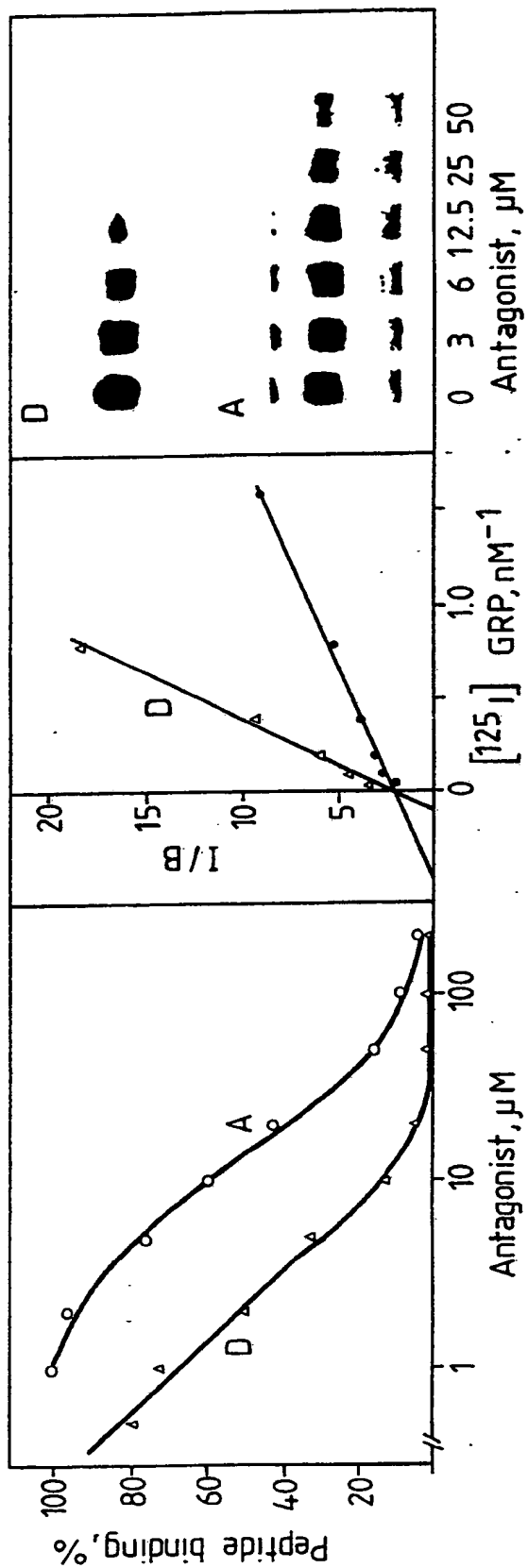


Fig.4.



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Fig. 2.

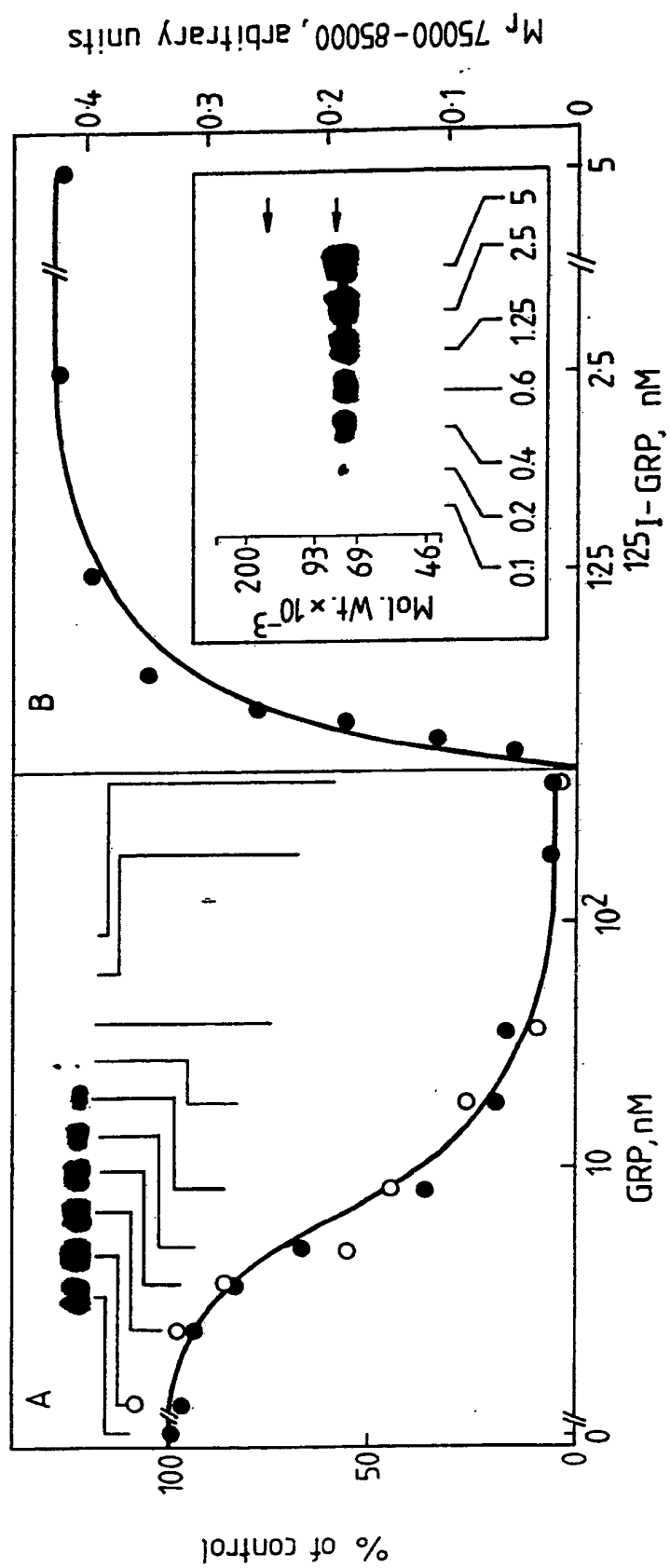
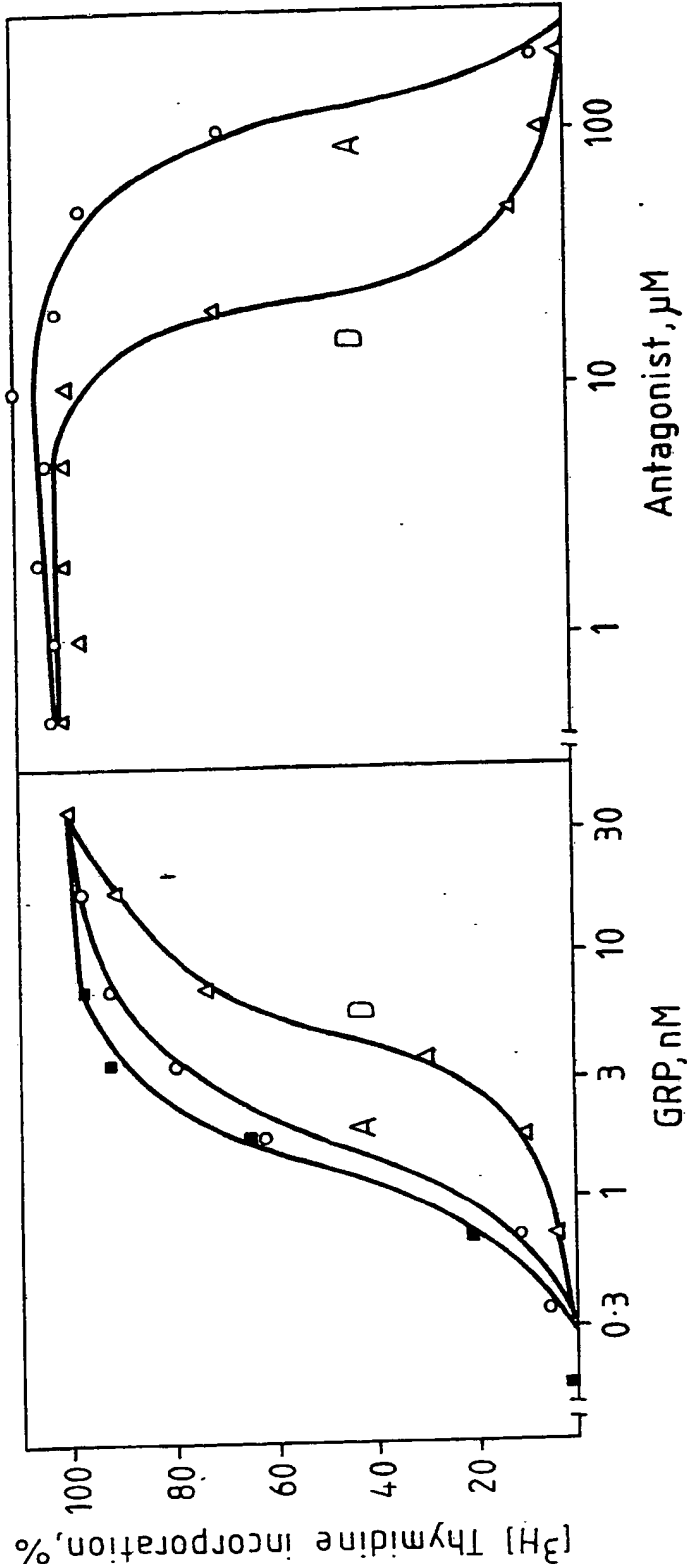


Fig. 3



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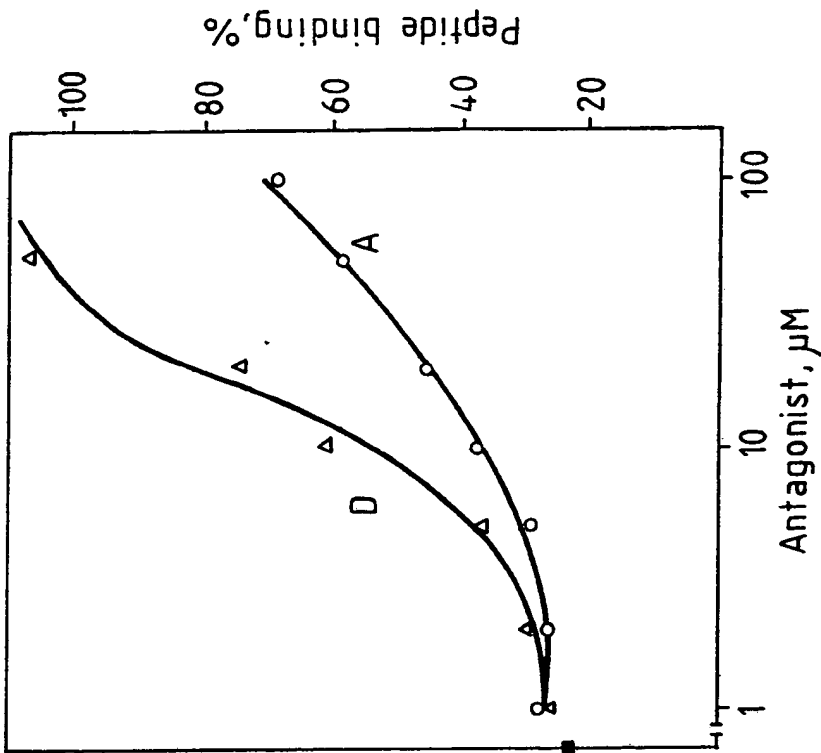
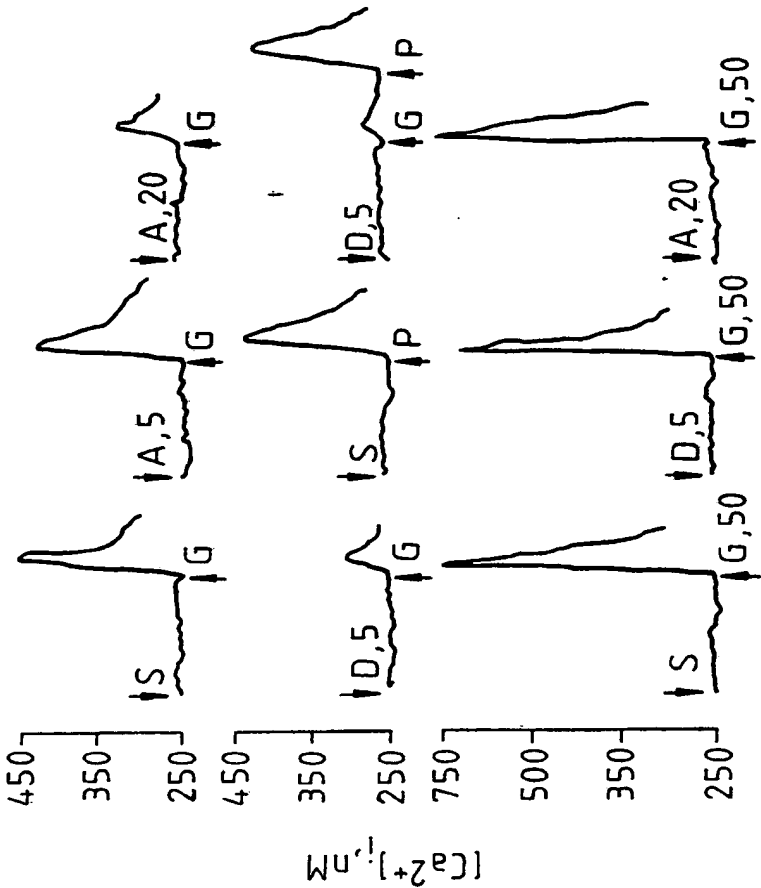
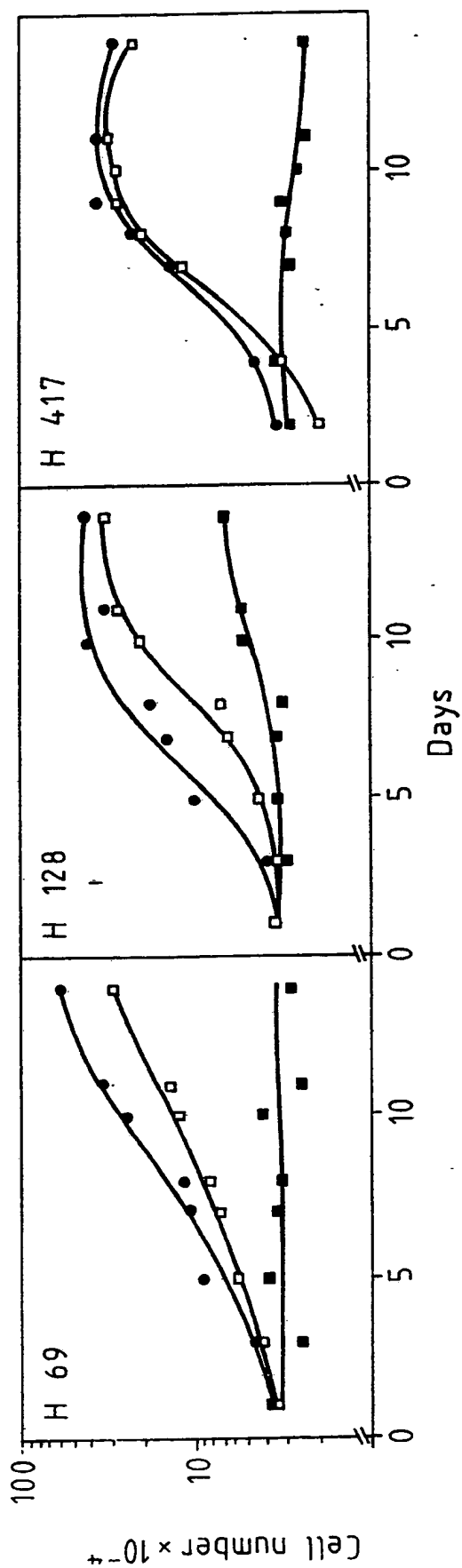


Fig.5



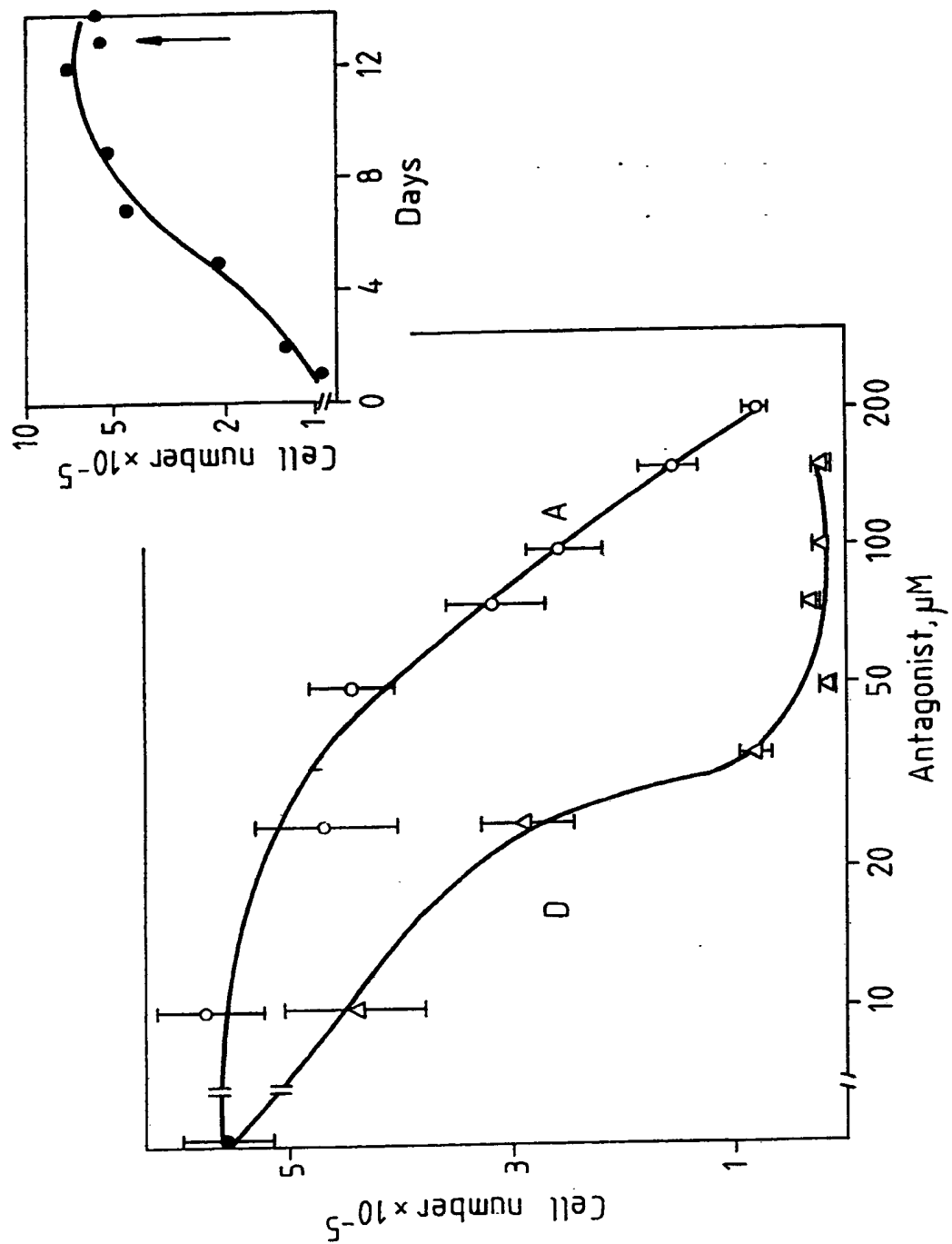
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Fig. 6



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Fig.7



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/GB88/00255</p> <p>(22) International Filing Date: 31 March 1988 (31.03.88)</p> <p>(31) Priority Application Numbers: 8707607 8727638</p> <p>(32) Priority Dates: 31 March 1987 (31.03.87) 25 November 1987 (25.11.87)</p> <p>(33) Priority Country: GB</p> <p>(71) Applicant (for all designated States except US): IMPERIAL CANCER RESEARCH TECHNOLOGY LIMITED [GB/GB]; 7-15 Lansdowne Road, Croydon, Surrey CR9 2PL (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : ROSENGURT, Enrique [GB/GB]; Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2 (GB). ZACHARY, Ian [GB/GB]; WOLL, Penellá [GB/GB]; Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A (GB).</p>	<p>(74) Agents: GOLDIN, Douglas, Michael et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5EU (GB).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p> <p>(88) Date of publication of the international search report: 22 February 1990 (22.02.90)</p>	
<p>(54) Title: GROWTH FACTOR RECEPTOR</p>		
<p>(57) Abstract</p> <p>A 75-85 Kd glycopolypeptide, capable of acting as a receptor for polypeptide of the bombesin type, is isolated from the surface of Swiss 3T3 cells. Certain antagonists and antibodies to the glycopolypeptide are described, the antagonists being of quite different structure to bombesin e.g. [D-Pro²]-spantide, [D-Phe⁵]-spantide and position 5 variants thereof. The antagonists and antibodies are of interest medically in that they are able to influence cell proliferation that occurs under the influence of the bombesin-like polypeptides.</p>		

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 88/00255

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C 07 K 15/00, A 61 K 37/02; G 01 N 33/574; C 07 K 7/06; A 61 K 39/395		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	C 12 P; A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	The Journal of Biological Chemistry; volume 262, no. 9, 25 March 1987, The American Society of Biological Chemists, Inc., (US), I. Zachary et al.: "Identification of a receptor for peptides of the bombesin family in Swiss 3T3 cells by affinity cross-linking", pages 3947-3950 see the whole document	1-4
Y	--	7-11,13,14
Y	Nature, volume 316, 29 August 1985, F. Cuttitta et al.: "Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer", pages 823-826 see page 826, column 1, lines 6-16 cited in the application --	7-11,13,14
X	Biological Abstracts, volume 83, D.M. Cirillo et al.: Receptor for bombesin with associated tyrosine .../.	1,7-11
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
29th June 1988	20. 09. 88	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	kinase activity", see abstract no. 66775, & Mol. Cell. Biol. 1986, vol. 6, no. 12, p. 4641-4649 --	
X	Proc. Natl. Acad. Sci, USA, volume 82, November 1985, I. Zachary et al.: "High-affinity receptors for peptides of the bombesin family in Swiss 3T3 cells", pages 7616-7620 see the whole document cited in the application --	11,13
P,X	The Journal of Biological Chemistry, volume 262, no. 23, 15 August 1987, The American Society for Biochemistry and Molecular Biology, Inc., (US), R.M. Kris et al.: "Identification of the bombesin receptor on murine and human cells by cross-linking experiments", pages 11215-11220 see the whole document --	1-4
X	Acta Chemica Scandinavica, volume B40, 1986, K. Folkers et al.: "Design and synthesis of antagonists of substance P", pages 295-302 see table I, nos. 1,4,10; table 2, nos. 1,3,6; table 7, no. 1 --	5
X	Biological Abstracts/RRM, no. 33040010, J.E. Layton et al.: "The effect of bombesin antagonists on the growth of small cell lung cancer SCLC", see the title, & Symposia on Molecular and Cellular Biology, Los Angeles, California, USA, January 17-23, 1987, J. Cell. Biochem. Suppl. 1987, vol. 0, no. 11, part A, p. 32 --	11,13
P,X	Proc. Natl. Acad. Sci, USA, volume 85, no. 6, March 1988, P.J. Woll et al.: "(D-Arg ¹ ,D-Phe ⁵ , D-Trp ^{7,9} ,Leu ¹¹)substance P, a potent bombesin antagonist in murine Swiss 3T3 cells, inhibits the growth of human small cell lung cancer cells in vitro", pages 1859-1863 see the whole document --	11,13
	./..	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET PCT/ISA/210

X

US, A, 4481139 (FOLKERS, K.)
6 November 1984
see table 1

5

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 12, 15, because they relate to subject matter not required to be searched by this Authority, namely:
see PCT - Rule 39.1 (iv):

Methods for treatment of the human or animal body by therapy,
as well as diagnostic methods.

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

Claims 1-4, 7, 10, 11, 13 and 14
Claims 5, 6, 13 and 14

for further information please
see PCT/ISA/206 mailed on
8th July 1988

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☒ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 8800255

SA 21551

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 13/09/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4481139	06-11-84	None	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET PCT/ISA/210

X US, A, 4481139 (FOLKERS, K.)
6 November 1984
see table 1

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V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

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3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

Claims 1-4, 7, 10, 11, 13 and 14
Claims 5, 6, 13 and 14

for further information please
see PCT/ISA/206 mailed on
8th July 1988

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4481139	06-11-84	None	